2010

A Guide to the Continuing Investigation of the Relationship between the Cytoskeleton and Cell Wall in Developing Buds of Physcomitrella patens

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I. Introduction

The mechanisms underlying an organism's ability to arrange its cells into a distinct form, and thereby also conferring a particular function to those cells, remains one of nature's enduring mysteries. The lure of this complex puzzle is the impetus behind this investigation into bud development. How a cell – which is fundamentally just a sac of chemicals, albeit a very complex and organized sac of chemicals – can bridge the gap between a *chemical* genotype and *physical* phenotype is a question as tantalizing and basic as the phenomenon of life itself. It is also a question that is equally challenging to answer. The goal of this manuscript is to explain the methods I have adopted to address this question, the progress made as of May 2010, and to outline the project components that remain to be completed.

In order to begin addressing the question of cell shape, one must first break it down into simpler questions that can be investigated in simple organisms. To do this I used the moss *Physcomitrella patens* as a model organism for investigating shoot morphogenesis. *Physcomitrella* serves as an excellent model organism because of its relative simplicity and user-friendly features. As can be seen in Figure 1, the dominant haploid phase has a relatively simple filamentous body plan on which leafy gametophores are generated. These gametophores start as a single cell that divides in a very orderly and predictable manner (Harrison et al. 2009) to produce a meristem-like bud with a single apical cell that continuously cleaves off cells to produce leaf-like structures in a spiral pattern (see Figure 2), thus serving as a simple model for the meristems of higher plants. It is the orderliness of the cell divisions in these buds that are of particular interest. What determines when and where a new cell is cleaved off from the apical cell to start new leaves? What internal and external signals orchestrate this fluid and rather elegant pageant? In order to explain this phenomenon one must look at what controls the growth of the individual cells.

Plant scientists have known for some time that the growth of plant cells is controlled by the orientation of the cellulose microfibrils of the cell wall (Taiz 1984). With their great tensile strength, microfibrils physically constrict the growing cell such that expansion occurs in a direction roughly perpendicular to microfibril orientation (Cosgrove 2000, see Figure 3). In a developing bud this process is slightly more complex because the cells do not grow in an obviously polar fashion as they do in filaments. However the principle remains: if you can orient

the microfibrils in such a way that reduces cell wall reinforcement in a particular direction, the cell will then expand and grow in that direction. This then begs the question: what controls the orientation of the microfibrils?

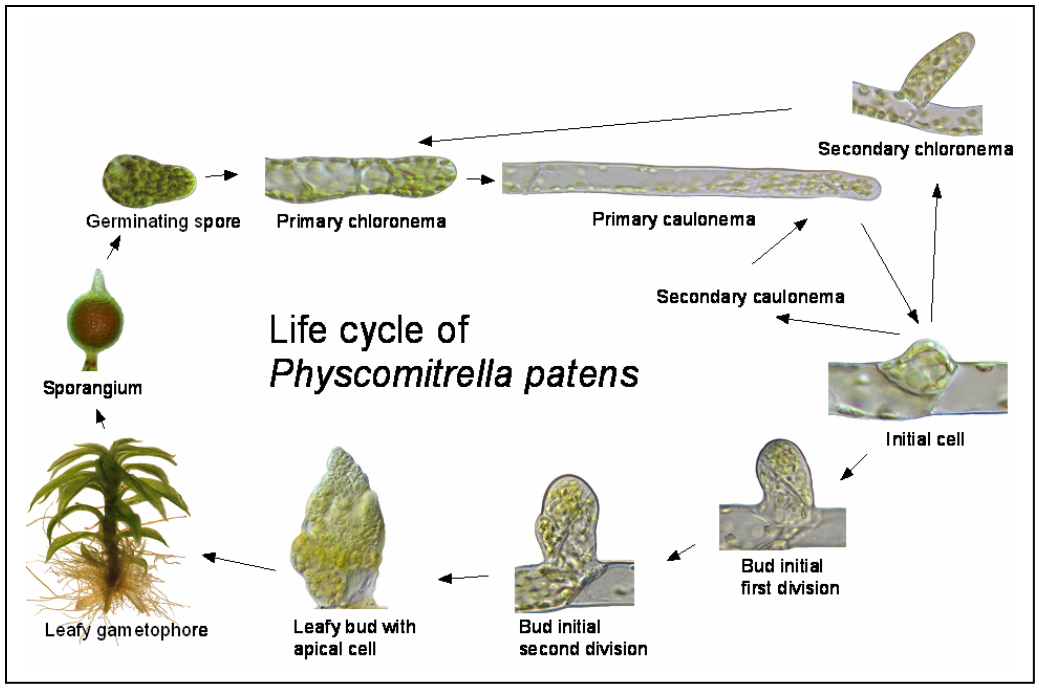

Figure 1. The *Physcomitrella* life cycle.

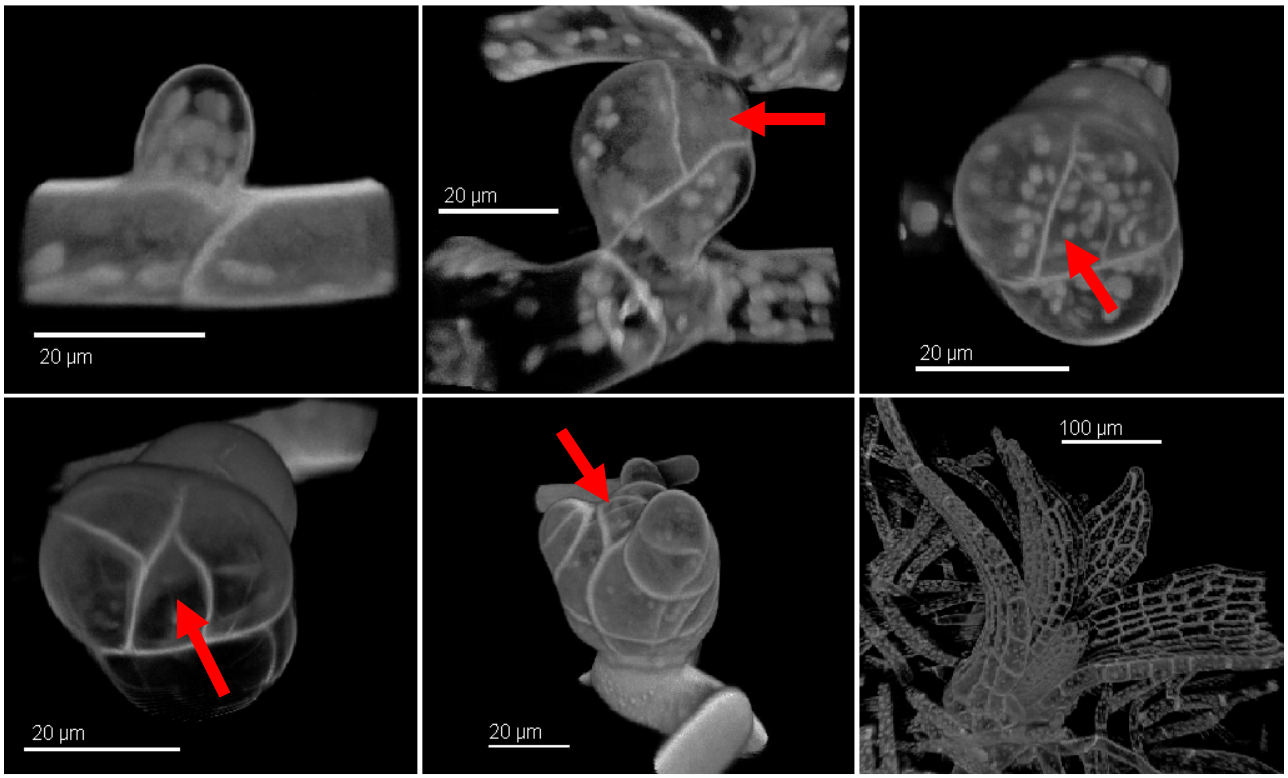

Figure 2. Confocal z-stack reconstructions of the stages of bud development. Arrows point to "apical" cells.

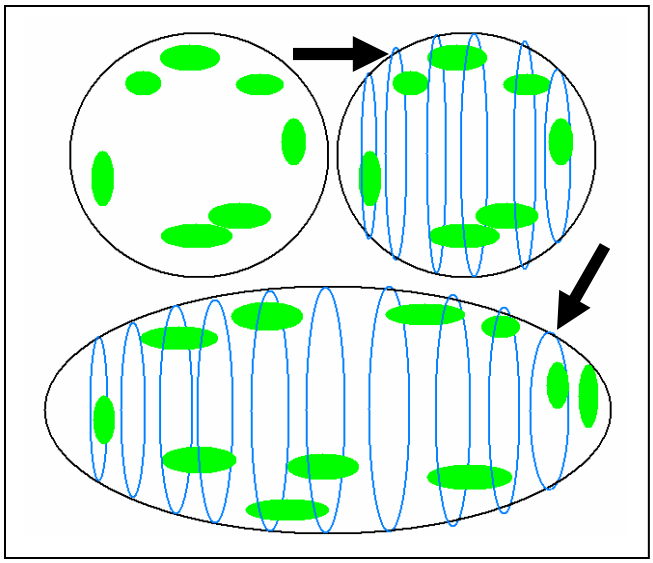

Figure 3. Principle of cell growth by cellulose reinforcement. Growth is roughly perpendicular to cellulose microfibril orientation

A number of experiments have demonstrated a functional association between the cellulose synthase terminal complexes and cortical microtubules of the cytoskeleton. These include experiments showing a loss of anisotropic growth when the microtubule cytoskeleton is disturbed with colchicine (Green 1962) as well as more recent experiments showing that cellulose synthase movement in the plasma membrane is strongly correlated with cortical microtubule orientation (Paradez et al. 2006, see Figure 4). The idea is that the microtubules act as bumper-like tracks that keep the cellulose synthase terminal complexes moving a specific direction. While this remains the dominant hypothesis, it has yet to be firmly demonstrated. Assuming that this "bumper" hypothesis is true, this just begs another question: what controls the orientation of the microtubules? What signals are directing the orientation of the microtubules such that the cell is able to spin its cocoon of cellulose and grow in an orderly fashion? The mechanism responsible for microtubule orientation remains to be elucidated.

There are a few possible mechanisms for the control of microtubule orientation. The first is that some sort of internal, chemical signal cascade could emanate from the genome, from other parts of the cell, or from some combination thereof which directly influences the chemistry and construction of the cortical microtubules. This could theoretically be accomplished through any number of methods, but the first that comes to mind is some sort of chemical gradient that establishes an axis of growth.

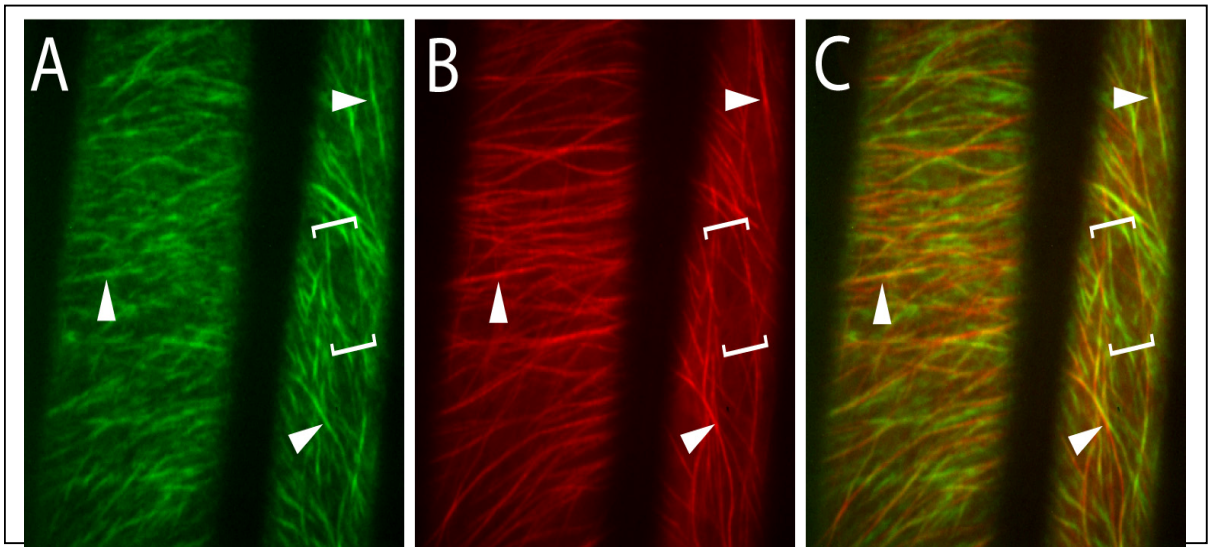

Figure 4. Confocal images of Arabidopsis root cells showing correlation of cellulose synthase movement (A) and microtubule orientation (B). Photo C is a composite image overlapping A and B. From Paredez AR, Somerville CR, Ehrhardt DW (2006). *Science* **312**, 1491-5.

Alternatively, microtubule orientation could be governed by biophysical forces acting on the cell. This is especially true for developing buds, in which growing leaf primordia could induce the initiation of the next primordium by causing the bud tissue to buckle in a regular pattern (Green 1999). Microtubules are known to respond to physical stresses and recent reports indicate that the dynamics of microtubules are somewhat self-organizing (Ehrhardt 2008) and that they are able to respond to the physical stresses of the meristem tissue in an coordinated way, possibly accounting for the patterns of shoot morphogenesis (Hamant et al. 2008).

While biophysical hypotheses appear promising, cortical microtubule arrangement is likely the result of a complex feedback between the cytoskeleton, the genome, physical forces, and other facets of cell chemistry. Included in this mix is the growth hormone auxin. By activating the extracellular enzyme expansin, auxin can initiate anisotropic growth by locally reducing cell wall reinforcement. So rather than the microtubules actively directing isotropic growth, they may just be reacting to physical forces induced by cell expansion and reinforcing that new orientation with the cellulose deposition they will subsequently guide. In this sense, both intracellular chemical signals and biophysical mechanisms could work in tandem to generate organized cell growth. The role of auxins and expansins in shoot morphogenesis was underscored by a report from Fleming et al. (1997) showing leaf-like structures emerging from and altering the phyllotaxy of meristems applied topically with expansin. It should be noted that *Physcomitrella* lacks polar auxin transport (Fujita 2008), thereby eliminating an otherwise confounding factor in this model system. Though such transport is not necessary for organized

bud development in *Physcomitrella*, this doesn't mean than auxin plays no role at all in organizing bud development through localized induction of cell expansion.

Nature has clearly created a complex mechanism for controlling cell shape and for coordinating organogenesis, with many components actively influencing each other. But science is slowly identifying the pieces of the puzzle and putting them together into a coherent picture. The goal of these experiments is to shed some light on this mystery by attempting to view the behavior of cortical microtubules and cellulose microfibrils over time in developing buds of *Physcomitrella patens*. For this experiment we are comparing wild type buds and the buds of a cellulose synthase mutant, *cesa5*, which have reduced cellulose deposition resulting in disorganized bud growth (Goss et al. 2010, see Figure 5). By comparing microtubule and microfibril behavior in these two lines, we may be able to glean to what degree biophysical forces affect microtubule organization. If in the mutant we see "organized" microtubules (like those seen in the wild type) despite reduced cellulose reinforcement, this would suggest that some internal, chemical signal is directing their organization and that biophysical influences play a secondary role. Conversely, if we see highly disorganized microtubules in the mutant, this would suggest that biophysical mechanisms are of primary significance in microtubule organization. However, given the current available information in this field, we may get some conflicting data. In this sense, this microscopy experiment is just the first part of a larger project in which more experimental tactics will need to be used.

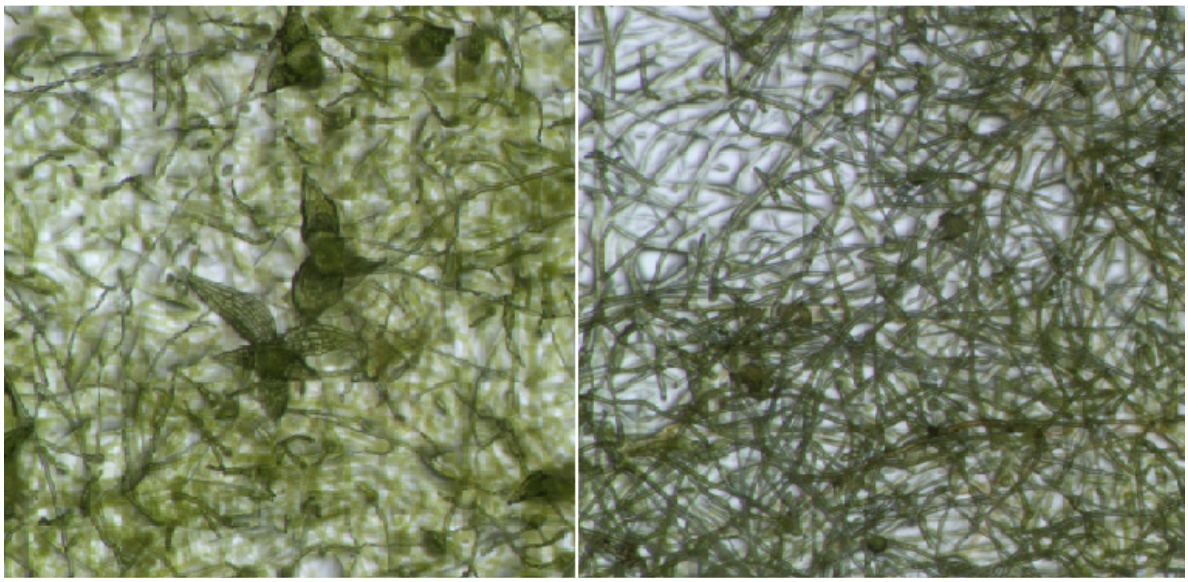

Figure 5. Wild type *Physcomitrella* leafy gametophores (left) and *cesa5* mutant buds (right). *cesa5* buds (indicated by arrows) are callus-like and disorganized due to reduced cellulose reinforcement from the lack of the CesA5 enzyme, which is expressed preferentially in the leafy gametophores.

II. Experimental Outline

1. Construct vector carrying GFP:tubulin gene

Status: Completed

2. Generate stable *CesA5* mutants

Status: Not started

3. Transform *Wt* and *cesa5* knock-out with GFP:tubulin in uj3

Status: Some lines established, more to be made

4. Optimize time-lapse confocal microscopy methods

Status: Completed

5. Optimize confocal microscopy methods for viewing microtubules and microfibrils

Status: Partially complete, more optimization required

6. Perform time-lapse confocal microscopy for viewing microtubules and microfibrils

Status: Not started

III. Experimental Procedures

1. Construct vector carrying GFP:tubulin gene

Two options were available for viewing microtubules: immunofluorescence and fluorescent protein tagging. Since immunofluorescence protocols kill the specimen and likely distort the delicate buds we're trying to examine, labeling the microtubules with green fluorescent protein (GFP) was the more desirable method.

I originally received a plasmid from the lab of Magdalena Benzanilla (University of Massachusetts, Amherst) that contained the sequence for GFP tagged onto the tubulin subunits of the microtubules. This plasmid was thought to be ready for transforming moss lines but restriction digests revealed that the plasmid was not large enough to contain all the necessary features (e.g. the selection cassette, etc.). However, the plasmid did contain the sequence for GFP:tubulin and was subsequently used as a template off of which to copy the sequence for further cloning into another vector.

Phusion DNA polymerase (Finnzymes, Espoo, Finland) was used in a PCR reaction (30 cycles of 98ºC denaturation for 8 s, 59ºC annealing for 20 s, 72ºC extension for 40s) to amplify

the GFP:tubulin sequence (See Table 1 for primers used). The 2.2 kb product was then cloned into the pENTR/D-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The GFP:tubulin sequence within the cloning vector was then sequenced to ensure there were no errors (see Table 1 for primers used). The GFP:tubulin sequence was then cloned into the uj3 expression vector with the ubiquitin promoter and a hygromycin resistance cassette. A complete sequence of the expression clone can be found in the Appendix and on the project's USB storage device. *E. coli* cultures containing this plasmid have been stored in 20% glycerol for later use. This vector can be linearized using *SwaI*, after harvesting and precipitating, in preparation for moss transformation.

Table 1. *Primers used for PCR and sequencing.*

Primer Name	Sequence	Expected Amplicon Size	Purpose
Act GFPENTR-F	CACCATGGTGAGCAAGGGCGAGGAG	2.2 kb	Phusion PCR (copying GFP:tubulin)
Act GFPENTR-R	TTACTTGTACCTCGAGTCAGT		Phusion PCR (copying GFP:tubulin)
M13 forward (Invitrogen)	GTAAAACGACGGCCAG	2.3 kb	Sequencing GFP:tubulin in pENTR/D-TOPO
M13 reverse (Invitrogen)	CAGGAAACAGCTATGAC		Sequencing GFP:tubulin in pENTR/D-TOPO
108 FW4	TACATTGCAGTGTAGCAGGCT	1,957 b	5' integration of vector
35int rev 2	ACCATCTGTGGGTTAGCGTCC		
108 rev 5	ACTGAAGGGTCGATGAGGTG	TBD (~1.7 kb)	3' integration of vector
Ubiq rev	To be designed		

2. Generate stable CesA5 mutants

Though I have been working with and have already transformed a few *cesa5-9* lines with the vector containing GFP:tubulin, these mutant lines have been noticeably losing their original phenotype over the course of a few months for unknown reasons. The mutant lines originally produced disorganized, callus-like buds (See Figure 5). Now they appear to produce buds with normal-like leaves and a spiral phyllotaxis. Unable to account for this phenomenon, the only way

to ensure that this microscopy experiment moves forward is to generate new, stable *CesA5* mutant lines that do not revert to the normal phenotype.

3. Transform Wt and cesa5 knock-out lines with GFP:tubulin

With the expression vector completed (see above), it now needs to be introduced into the *wild type* and *cesa5* genomes. As explained above, selection marker is for hygromycin resistance. When properly integrated, the moss should express the GFP:tubulin sequence throughout the organism, including buds, making the microtubules visible with fluorescence microscopy. Though *wild type* and *cesa5-9* lines have been transformed and some stable transformants have been recovered, more transformations should be performed to ensure the acquired lines have properly integrated the DNA and adequately express the GFP:tubulin gene. See Table 1 for the primers to be used in testing for 5' and 3' integration of the vector.

4. Optimize time-lapse confocal microscopy methods

To view cell divisions, and thus the changing microtubule and microfibril behavior, I used confocal laser scanning microscopy (CLSM) to generate 3D images of *Physcomitrella* buds at regular time intervals. I spent a few months figuring out what methods to use to retrieve the best images without inhibiting normal bud development. The following procedure can be used to capture and compare the cell divisions within the *wild type* and *cesa5* buds:

Preparing the Specimens

For this experiment, the moss will be subjected to CLSM while growing directly on a thin layer of agar on the microscope slide. After trying several other methods I discovered that this protocol is best because it keeps the specimen fixed in place and allows it to grow with minimal stress. To prepare *wild type* and *cesa5* specimens, first prepare protoplasts of each line, plate in petri dishes containing PRMB overlain with cellophane for 3-4 days, then transfer the cellophane with protoplasts to petri dishes containing BCD medium to induce gametophore development. Buds should start forming by day 6 after transfer for BCD. At this time, prepare slides with wells made from electrical tape by placing 5-8 layers of tape on one side of the slide and then carving out a square-shaped well in the middle of the tape with a razor knife (see Figure 6, some slides should already be made). Clean the wells of residual adhesive with 70% ethanol. Sterilize the

wells by cleaning with additional 70% ethanol. Carefully pipet melted solid BCD medium into the wells until the agar is about level with the top of the wells and let cool. As the medium cools it will contract to make a slight depression in the well. Once cooled, the slides are ready for culturing moss.

To transfer the moss from petri dishes, pipet approximately 200 µl of sterile water onto the wells, creating a large droplet of water. Carefully extract moss colonies from the petri dishes using fine-tipped tweezers and place them in the droplet of water. When released, the moss should spread out evenly and float in the droplet of water (See Figure 6). When finished loading the droplet with moss colonies (about 3-4, depending on the size), carefully insert the pipet tip into the droplet under the moss and slowly draw out the liquid. This will lower the moss evenly and cleanly onto the agar in the well (See Figure 6). When finished, place the slides in a humid chamber (usually crafted from a petri dish), place in an incubator, and allow the moss to grow into the agar for 24 hours. Nourish the moss with small amounts of liquid BCD every 12 hours to avoid drying out.

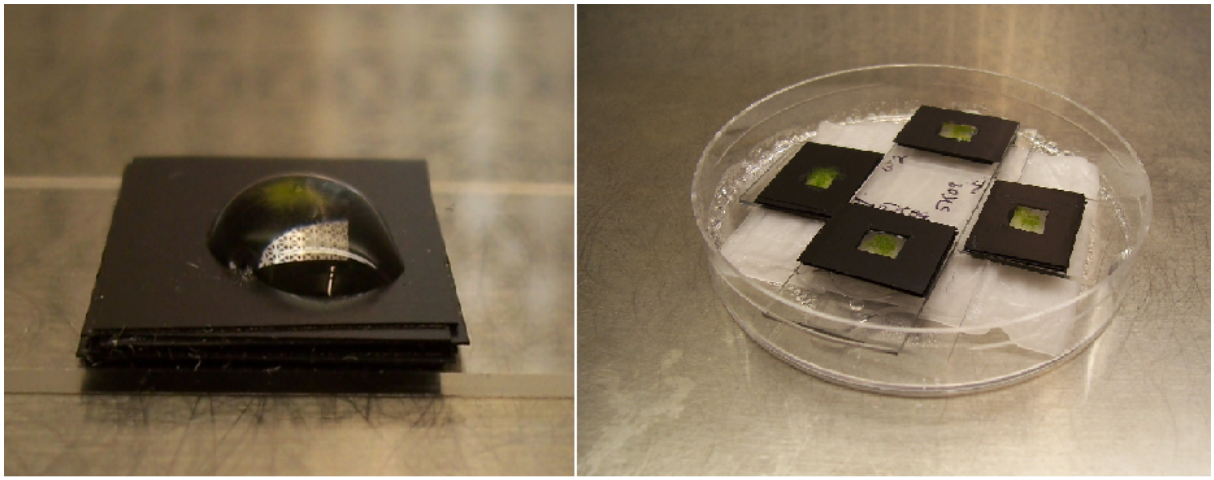

Figure 6. Loading the moss into the wells on a microscope slide requires placing colonies in a droplet of water (left). Removing the liquid with a micropipetor lowers the moss cleanly onto the agar without distorting the specimen. The moss is then allowed to grow into the agar while housed in a humidified petri dish (right) kept in a 25ºC incubator.

Performing CLSM

CLSM is an extremely valuable microscopy method that allows investigators to acquire a level of detail and clarity not available through other microscopy methods. CLSM works by focusing a laser onto a single plane of focus about 1 µm thin to excite a dye or protein that fluoresces, thereby labeling a structure of interest. After acquiring a stack of these images, one

after another through a specimen, they can then be reassembled in a computer program to create a 3D image of the specimen. In our case, the specimen of interest is a developing bud.

To perform the CLSM, retrieve the moss cultures on the slides prepared 24 in advance. Place the slides in the holder on the stage and pipet approximately 200 µl of staining solution (in this case, 30 µg/ml propidium iodide to stain the cell walls) on top of the specimens to create a droplet. The moss should not float after growing into the agar for 24 hours. Using the 40x water immersion lens, raise the microscope stage so that the water droplet makes contact with the lens. Using brightfield microscopy, scan for buds. Avoid buds that are thickly entangled in other filaments – these usually don't stain well. Also avoid buds that are noticeably red in color – these have taken up the stain inside the cell because they have died. These tricks and others become obvious as you perform the CLSM.

When you find a bud suitable for CLSM, acquire a z-stack every 12 hours according to the instructions you receive from an experienced user, using the GFP configuration (488 nm excitation, 510 nm emission). In between imaging sessions return the slides to the humid chamber in an incubator and remove the staining solution using a pipetor. A 24-hour interval is probably sufficient for capturing noticeable cell divisions. Regardless of the time interval, be sure to record the "coordinate" location of each bud using the micrometer on the two axes of the microscope stage. This is the best way to find the same bud when you return each time to acquire new images. Be sure to use a log to track the development of each bud (a model log is provided in the Appendix). To ensure that you get a good series of images, I recommend taking z-stacks of about 10 different buds in each line you are tracking. Some buds will inevitably die during the course of the experiment, others will turn out to be blurry or indecipherable, and in the end only about 3/10 buds will be satisfactory for collecting data. Since you will be taking lots of images, I recommend naming each bud and creating a folder within the computer database for each so that you can more easily keep track of which image belongs to which bud.

The following tips will help acquire better images:

- To speed up the scan time, crop the image using the zoom feature so that you are scanning only the bud and not filaments or blank space around it.
- Adjust the scan speed to the maximum speed that still gives you good resolution.
- Use the averaging feature to cut out "noise" and create smoother images.

- Overlap consecutive slices in a z-stack such that the next slice will re-scan half of the previous image. To do this, set the slice interval to one-half the distance of the optical slice thickness. This helps to reduce the visibility of lines between slices when assembling the z-stack into a 3D image.

Once you have acquired about 4-6 z-stacks of developing buds, transfer the files to a flash drive (with plenty of space, >2 GB) and use the Axiovision software to assemble the z-stacks into 3D images. Use the settings panel to adjust brightness and contrast; some images may initially seem useless but when you adjust these parameters the image may turn out to be quite informative. Images that have been acquired using this method can be seen in Figure 7.

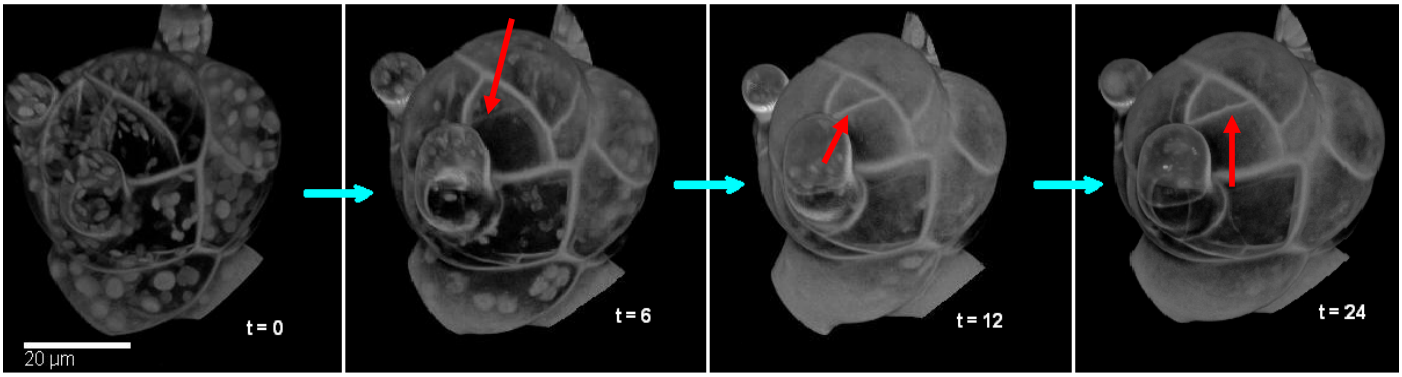

Figure 7. Time-lapse confocal images showing the cleavage of the apical cell over a 24-hour period. The red arrows show the new cell wall being deposited.

5. Optimize confocal microscopy methods for viewing microtubules and microfibrils

Ideally, we would be able to view microtubule and microfibril behavior over time using the same time-lapse method described above. However, as of now, the nature of *Physcomitrella* and the University's equipment present some challenges. I was able to begin optimizing the methods for viewing microtubules and microfibrils using a wild type line already transformed with GFP:tubulin from the lab of Luis Vidali (Worcester Polytechnic Institute). This transformant does not express GFP:tubulin very well in the buds (which is why we need to transform wild type lines with a vector gene driven by the ubiquitin promoter), but I was able to use it to begin determining the parameters for this kind of microscopy. In this section I share what I have learned thus far and provide pictures showing the kinds of images I was able to acquire.

For this type of microscopy I intended to use the same water-immersion method described above to track the changes in microtubule and microfibril behavior. However, the 40x water immersion lens does not have a high enough numerical aperture to allow enough light to enter the lens, thereby limiting the level of visible detail. In response, I used a traditional wet mount with a coverslip and used the 63x oil immersion lens instead. This means that we cannot view individual buds as they develop over time, but rather must compare different buds at what we think are similar stages.

To view the cellulose microfibrils I stained the GFP:tubulin-transformed moss in a solution of 0.01% pontamine fast scarlet (S4B) for 30 minutes in a microcentrifuge tube and then rinsed with water as described by Anderson et al. (2009). I then prepared a wet mount as described above and performed CLSM using the two-channel FITC/Rhodamine configuration (GFP: 488nm excitation; S4B: 543nm excitation). The goal is to get images like those produced by Paradez et al. (2006), even though they used different methods (See Figure 4)

For this type of microscopy, the challenge to overcome is that of acquiring higher resolution. Both the GFP and the microfibrils are visible but not with the degree of resolution I hoped for (See Figure 8). To acquire higher-resolution images the scan speed must be set to a very slow speed, and the averaging feature also slows down the scan speed. The problem with this is that the specimen is subjected to intense light for a long period of time, causing both the GFP and the S4B stain to quickly fade. This limits the number of images that can be taken and thus limits the size of a z-stack. So it appears that we cannot get a 3D reconstruction of an entire bud showing its microtubule and microfibril orientations. However, it should be possible to get partial images by scanning thinner slices of the cell walls (See Figure 9). Ultimately, the challenge is to balance the desired level of resolution with exposure time.

While the microtubules labeled with GFP are readily visible with comparatively good resolution, the cellulose microfibrils were not so readily visible (See Figure 8). A thin patchwork of fibers can be made out in the best images, which are thin tangential slices through the cell wall, but these microfibrils are not nearly as distinct and pronounced as those seen in *Arabidopsis* using the same dye and method (Anderson et al. 2009). Pontamine fast scarlet fluoresces preferentially in the presence of cellulose, but it also binds to other components of the cell wall. Since *Physcomitrella* is a small and relatively fast-growing organism, its cell wall doesn't have the thick bundles of cellulose microfibrils that a higher plant like *Arabidopsis* has.

Thus, *Physcomitrella*'s microfibrils are not as visible because they are individually thin and tightly-packed, causing a haze of fluorescence that drowns out the signal from individual fibers.

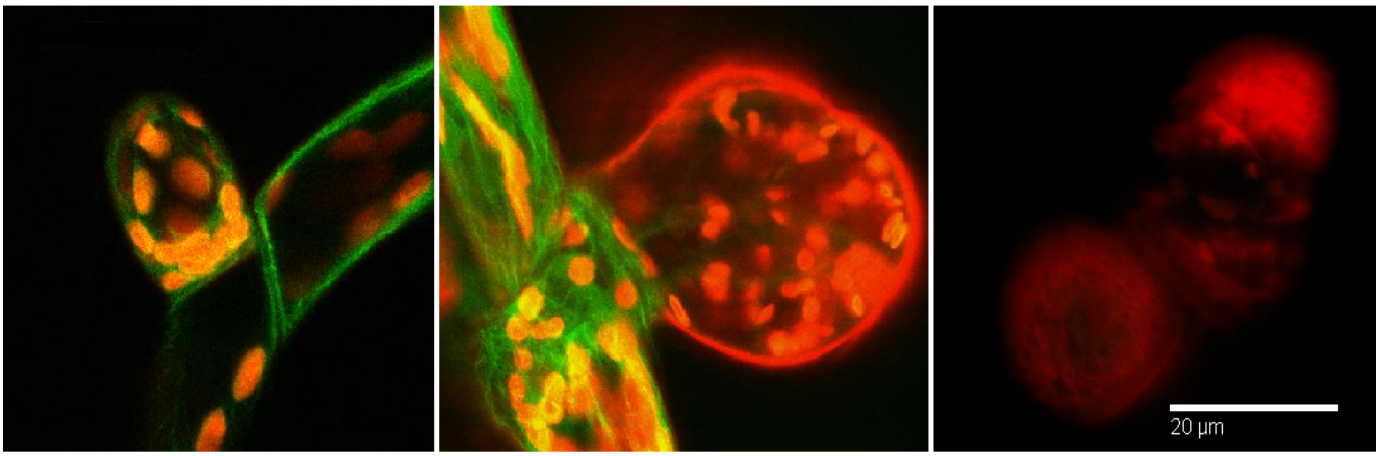

Figure 8. CLSM images of wild type moss transformed with GFP:tubulin and cell walls stained with pontamine fast scarlet. Microtubules (green) and microfibrils (red) are both visible, but microfibrils have less resolution than microtubules. Microfibrils are best viewed in a thin z-stack taken through a tangential section of the cell wall, as in the image on the right where a thin patchwork of fibers can be seen.

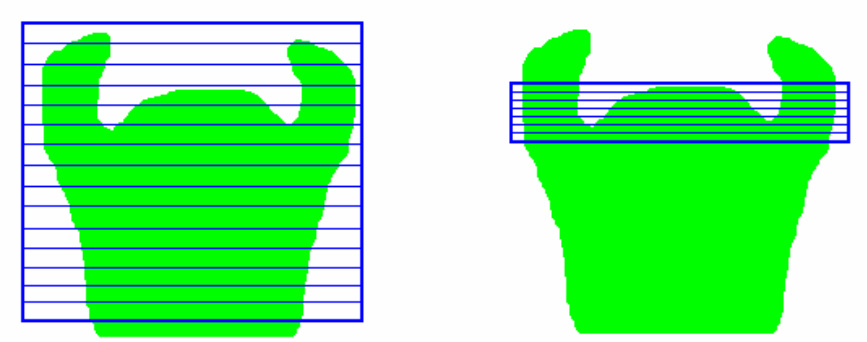

Figure 9. Large z-stacks with thick slices that capture the entire bud (left) expose the specimen to intense light for too long and have reduced microtubule and microfibril resolution. Resolution can be improved by taking fewer but thinner slices through the most critical parts of the bud.

This particular microscopy experiment presents a number of different, but inter-related challenges. Some suggestions for combating these issues include:

- Using a greater image pixel width and height to gain greater resolution.
- Investigating why the GFP in particular is bleaching out so quickly under the laser.
- Using a different method for viewing microfibril behavior. Try different stains, or label the cellulose synthases with YFP and take time-lapse images as done by Paradez

et al. (2006). Alternatively, microfibril behavior could be ignored since we are more interested in microtubule behavior.

- Purchasing a water immersion lens with greater numerical aperture and greater magnification to be able to track individual buds as the develop.
- Select buds that are not entangled in other filaments. The fluorescence from other filaments tends to drown out the signal from the bud.

With a few more modifications and the use of transformants from Part 3 of this experiment, it is very likely that we can acquire high resolution images of at least the microtubule behavior in developing buds.

6. Perform time-lapse confocal microscopy for viewing microtubules and microfibrils

This final part of the experiment is the actual data-collecting experiment. Once all of the microscopy methods have been optimized and we get stable transformants that express the introduced GFP:tubulin gene, we can actually perform the experiment and view the behavior of microtubules and possibly microfibrils as Physcomitrella buds grow. At that point, we can begin to infer the role of biophysical forces in organized cell growth.

IV. <u>Concluding Remarks</u>

When I first conceived this project in my mind, it seemed that it was nothing more than a simple microscopy experiment that just might tell us something about the way nature works. While this is still, in principle, a simple microscopy experiment (it's not exactly the human genome project), it became more involved and time-consuming as I broke it down into its component steps. To do this project correctly and completely I would likely need to spend the better part of a year working on it – more than the few months I allotted. However, I think that I made substantial progress in that short amount of time while learning valuable lessons about the scientific process. With another semester's worth of work the project can be near complete. As simple as this project is, I think it shows great promise for teaching us about some of nature's puzzles. Finally, I think *Physcomitrella* shows great promise for investigating shoot morphogenesis and that this project will naturally kindle ideas for countless future projects.

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APPENDIX

Expression Clone (Complete Sequence)

Start codon for the GFP:tubulin gene highlighted in green; stop codon in red.

TATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACT
TAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
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Confocal Microscopy Log

Specimen	Time					
Wild Type	Coordinates	t = 0	t = 12	t = 42	t = 36	t = 48
Alpha						
Beta						
Gamma						
Delta						
Epsilon						
Zeta						
Eta						
Theta						
Iota						
Kappa						
cesa5	Coordinates	t = 0	t = 12	t = 42	t = 36	t = 48
Alpha						
Beta						
Gamma						
Delta						
Epsilon						
Zeta						
Eta						
Theta						
Iota						
Kappa						